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Distribution of mRNA encoding the inwardly rectifying K⁺ channel, BIR1 in rat tissues

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Abstract The distribution of mRNA encoding the inwardly rectifying K⁺ channel, BIR1 [1] was investigated in rat tissues, and a comparison made with the expression of related genes rK_{ATP} and GIRK1 using the reverse transcription-polymerase chain reaction (RT-PCR). This showed BIR1 to be expressed in all areas of the brain examined, in the eye but not in any other peripheral tissue. This pattern was distinct from rK_{ATP} and GIRK1. Additional *in situ* hybridisation studies of the central expression of BIR1 demonstrated high levels of BIR1 mRNA in the hippocampus, dentate gyrus, taenia tecta and cerebellum and at lower levels in the cortex, habenular nucleus, olfactory bulb, primary olfactory cortex, thalamus, pontine nucleus and amygdaloid nucleus.

Key words: Inward rectifier; BIR1; rK_{ATP}; GIRK1; Potassium channel; mRNA; Hybridisation, *in situ*

1. Introduction

A number of members of a gene family have now been described that encode inwardly rectifying K⁺ channels [2]. One subfamily of these, designated Kir 3.0, encode proteins that, when heterologously expressed, give rise to K⁺ channels modulated by G-protein activation [3–5] and by the presence of intracellular ATP [6,7]. A recent addition to this list is a gene isolated from a rat insulinoma cell line (RINm5F), known as BIR1 [1] which has 72% and 53% identity to rK_{ATP} and GIRK1, respectively and is virtually identical (98%) to GIRK2, a brain inward rectifier K⁺ channel also modulated by G-protein activation [5]. Expression of the BIR1 sequence in *Xenopus* oocytes demonstrated, under voltage clamp recording, the presence of an inwardly rectifying potassium channel. Although the nucleotide sequence of BIR1 closely resembles that of rK_{ATP} the expressed protein gives rise to inwardly rectifying potassium currents that are not blocked by ATP [1]. Various putative regulators in the generation of inward rectifier channels have distinct physiological and pharmacological properties [2,8]. Recent studies also clearly indicate that various members of this gene family co-assemble and form functional heteromultimeric channels [9–12]. However, it is still unclear how inwardly rectifying K⁺ channels are formed and what relation there is between the cloned proteins *in vivo*. Therefore to elucidate further the relationship between these genes we have examined the distribution of BIR1 mRNA in the rat, and compared its distribution with rK_{ATP} and GIRK1 mRNAs using the reverse transcription-polymerase chain reaction (RT-PCR). We have

also performed a more detailed study of the expression of BIR1 in rat brain using *in situ* hybridisation.

2. Materials and methods

2.1. Reverse transcription PCR (RT-PCR)

Sprague–Dawley rats (8–12 weeks), maintained in a 12 h light–dark cycle with free access to laboratory rodent chow were killed by cervical dislocation. Tissues were rapidly dissected out and snap frozen in isopentane cooled by solid CO₂ and stored at –70°C. Total RNA was isolated from samples of rat tissue and the insulin-secreting cell line, CRI G1, by acid guanidium thiocyanate/phenol/chloroform extraction [13]. Total RNA (10 µg) was reverse transcribed in the presence of an anchored oligo(dT)-primer, T₁₇(A,G,C), using M-MLV reverse transcriptase (Gibco BRL, Paisley, Renfrewshire, UK) to manufacturers' recommendations. PCR primers were synthesised from the BIR1, rK_{ATP} and GIRK1 cDNA sequences, (see Table 1 for details). PCR primers for β-actin were used to assess the integrity of RNA samples; to guard against false negatives. Primers for sucrase-isomaltase were used to ensure specific amplification from RNA, in this case safeguarding against false positives due to amplification of genomic DNA. All primers were synthesised on an Applied Biosystems 394 DNA/RNA (Perkin Elmer, Warrington, Cheshire, UK) and all PCR reactions were carried out on a Hybaid Omnigene (Hybaid, Teddington, Middlesex, UK). Following optimisation of PCR conditions to obtain a single band, all products were subjected to a restriction digest in order to verify the identity of PCR product. 15 µg of amplified cDNAs were digested to completion at 37°C for 1 h with the endonucleases *Hae*III and *Aha*I (Biolabs, New England, USA). Digestion products were then separated on a 3.5% MetaPhor agarose gel (FMC BioProducts, Rockland, USA). The same single-stranded cDNA product produced by the reverse transcription of total RNAs isolated from each tissue sample was used to analyse the expression pattern of these genes. RT product equivalent to that produced from 50 ng of total RNA was used for subsequent PCR amplification (30 cycles). Each PCR cycle consisted of denaturing at 94°C for 45 s, annealing at 64°C with rK_{ATP}, GIRK1, sucrase-isomaltase and β-actin primers and 67°C with BIR1 primers for 45 s, and extension at 72°C for 1 min. The reaction mixture contained; 0.5 mM dNTPs, 1.2 U AmpliTaq DNA polymerase (Perkin Elmer), 0.5 U Perfect Match (Stratagene, Cambridge, Cambs., UK), 1 × PCR buffer (3.5 mM Mg²⁺, pH 8.8). PCR products were separated on a 2.5% agarose gel (Gibco BRL).

2.2. *In situ* hybridisation

Rat brains were rapidly dissected out and snap frozen in isopentane cooled by solid CO₂ and stored at –70°C. To assess comprehensively the central distribution of BIR1 mRNA sagittal, horizontal and vertical brain sections were cut (10 µm) encompassing most major regions. Cryostat sections were thaw-mounted onto (poly)-L-lysine-coated slides, fixed in 4% formaldehyde and stored in 95% ethanol at 4°C. The basic method used for *in situ* hybridisation has been described previously [14]. Briefly, sense and anti-sense oligodeoxyribonucleotide probes complementary to the rat BIR1 channel were synthesised; the sequence of the anti-sense probe (5'-GTC AGG ACT GGT GTG AAC CGG TAA CCC CAC AGG ATC TCA CTG GTG-3' nucleotides 984–928) was unique to the rat BIR1 sequence when examined against all known rodent sequences. Both sense and anti-sense probes were labelled with α³²S]dATP (>1000 Ci/mmol, Amersham Int. plc, Amer-

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sham, Bucks., UK) using terminal deoxynucleotidyl transferase (Pharmacia, Milton Keynes, Bucks., UK) at 32°C for 1 h. Labelled probe was applied to sections in hybridisation buffer containing 4 × standard sodium citrate (SSC) and 50% deionised formamide. The slides were incubated overnight at 42°C before being washed in 1 × SSC containing 0.1% sodium thiosulphate for 1 h at 55°C and dehydrated through alcohol. Slides were exposed to X-ray film (Kodak, Biomax, Rochester, NY, USA) for 7 days, dipped in Ilford K5 emulsion (Amersham), and stored desiccated at 4°C for 2 weeks. After development, brain sections were stained with hematoxylin and eosin and mounted with a coverslip.

2.3. Northern blot

Poly(A)⁺ RNA was selected using a Fast Track mRNA isolation kit (Invitrogen, San Diego, USA), separated by electrophoresis on a 1.2% formaldehyde agarose gel prior to transfer onto Hybond-N membrane (Amersham). RNA was fixed to the membrane by UV irradiation and molecular mass markers were made visible by ethidium bromide staining. After prehybridisation at 42°C for 1 h, the blot was hybridised at 42°C for 2 h with the anti-sense probe, end labelled with [³²P]dATP (6000 Ci/mmol; Amersham) using terminal deoxynucleotidyl transferase (Pharmacia), in QuikHyb hybridisation buffer (Stratagene). After washing in 1 × SSC/0.1% sodium dodecyl sulphate at room temperature for 30 min and then at 55°C for 30 min, the blot was wrapped in Saran wrap and exposed for 2 weeks to Fuji RX X-ray film (Genetic Research, Felsted, Essex, UK) at –70°C in a X-ray cassette containing intensifying screens.

3. Results

RT-PCR has been used to investigate the expression of the recently cloned inwardly rectifying K⁺ channel BIR1 in rat tissues, and compared with the related genes rK_{ATP} and GIRK1. To ensure reproducibility and the physiological relevance of this work several precautionary steps were included. Firstly, all PCR amplifications have been carried out on the same cDNA samples; total RNA samples having been reversed transcribed using an anchored oligo(dT)-primer. Secondly, amplification of β-actin cDNA confirmed the integrity of the RNA

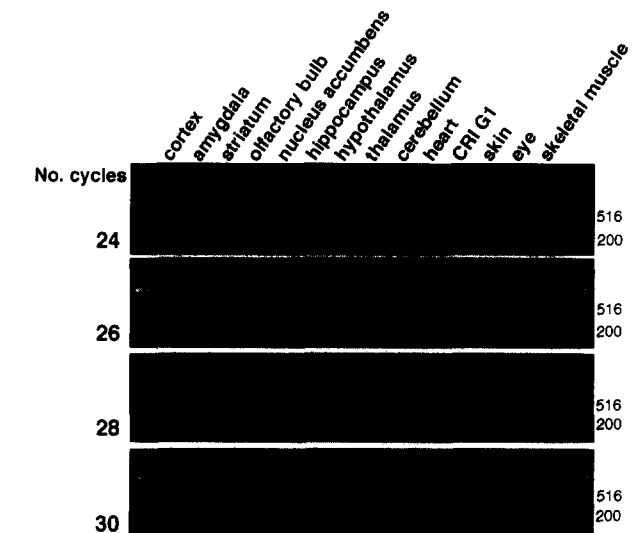


Fig. 2. Relative abundance of BIR1 mRNA. RT-PCR was performed on cDNA products and the PCR reaction mix sampled following 24, 26, 28 and 30 cycles of amplification and separated on a 2.5% agarose gel. Results suggest that expression in the central nervous system is highest in the hippocampus, cerebellum and thalamus.

and its subsequent reverse transcription for each tissue sample, guarding against false negatives. Finally, PCR amplification using sucrase-isomaltase primers was performed on all cDNA samples to ensure that the samples were not contaminated with genomic DNA and that the PCR conditions were not over-sensitive. Sucrase-isomaltase expression is restricted to the epithelium of the small intestine [15] and therefore no amplification product should be observed in other tissues, safeguarding against false positives.

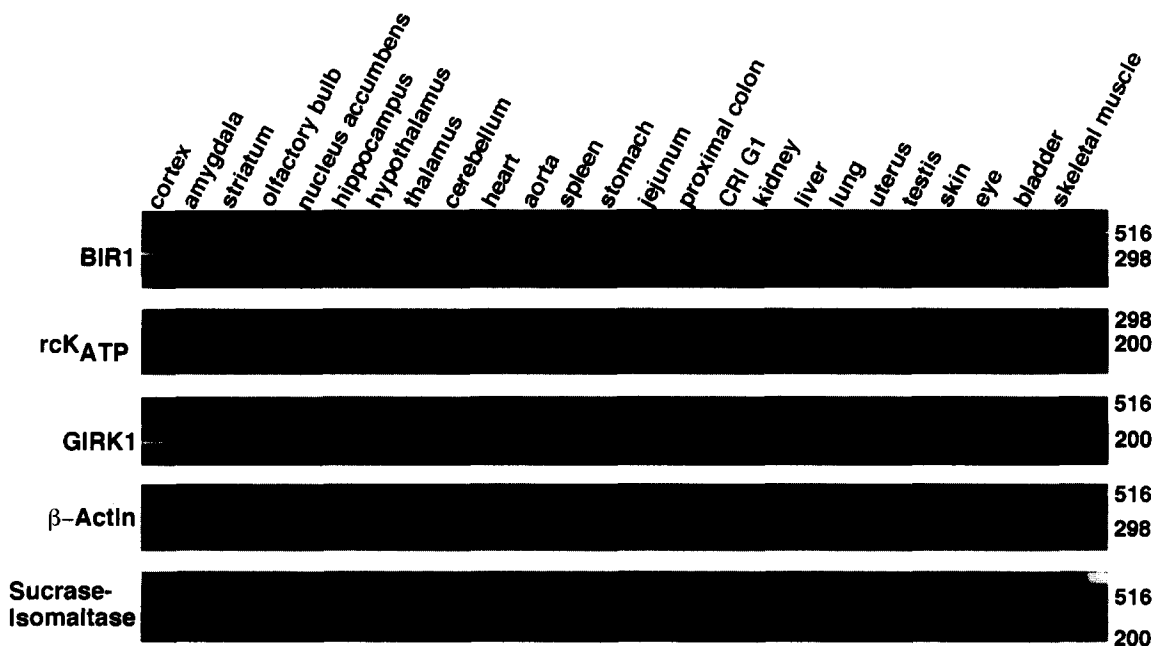


Fig. 1. Tissue distribution of the BIR1, rK_{ATP} and GIRK1 mRNAs as examined by RT-PCR. Total RNA extracted from a range of rat tissues and insulinoma cell line, CRI G1, was reverse transcribed in the presence of an anchored oligo(dT) primer and M-MLV. cDNA products were then subjected to 30 cycles of PCR amplification using forward and reverse primers specific to each cDNA sequence. Amplification of β-actin and sucrase isomaltase cDNA serve as controls. PCR products were separated on a 2.5% agarose gel.

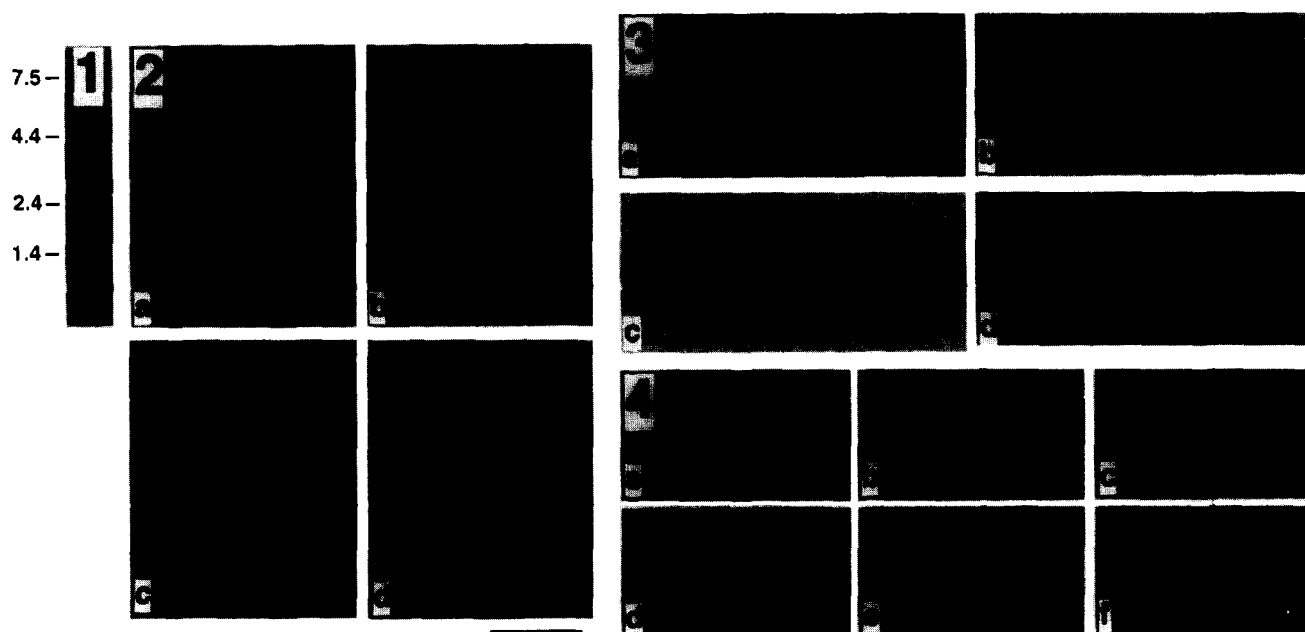


Fig. 3. Expression of BIR1 in the rat brain. (1) Northern blot analysis of the anti-sense oligonucleotide probe complementary to BIR1 mRNA. Hybridisation to poly(A)⁺ RNA (10 μ g) extracted from whole rat brain demonstrated the probe to bind to RNA of 1.4 kb. X-Ray autoradiographs of horizontal (2), sagittal (3) and vertical (4) sections of rat brain after hybridisation of the BIR1 anti-sense probe. Brain areas showing specific labelling are indicated: AM, amygdaloid nucleus; CB, cerebellum; CT, cortex; DG, dentate gyrus; HIP, hippocampus; OB, olfactory bulb; PO, primary olfactory cortex; PN, pontine nucleus; TH, thalamus; TT, taenia tecta, HB, habenular nucleus. Scale bar=0.5 cm.

Following optimisation of the reaction conditions all primer pairs gave a single cDNA band of the expected size. A restriction digest of the cDNA product verified that the RT-PCR had amplified specifically the mRNAs of interest (data not shown). RT-PCR was then carried out on a range of rat tissues and rat insulinoma cells, CRI G1, similar to the cells from which the BIR1 was recently cloned [1] (Fig. 1).

BIR1 expression was found throughout the brain; strong amplification occurring from the cortex, olfactory bulb, hippocampus, hypothalamus, thalamus and cerebellum cDNAs, whilst moderate levels of PCR product were obtained from the amygdaloid nucleus, striatum and the nucleus accumbens. Amongst the peripheral tissues, BIR1 expression could only be detected in the eye. However, as predicted CRI G1 cell cDNA also gave a signal by RT-PCR (Fig. 1). Use of both forward primers with the reverse primer gave identical results (data not shown).

To investigate more closely the distribution of BIR1 mRNA in areas where PCR products appeared after 30 cycles of amplification, further RT-PCR studies were carried out. In these studies, samples were taken out and analysed following 24 cycles of amplification and then following every subsequent two rounds of amplification (Fig. 2). cDNAs from the heart, skin and skeletal muscle served as negative controls. Since the appearance of the RT-PCR product is proportional to the amount of BIR1 mRNA present in the initial sample (data not shown), these results suggest that expression in the brain is highest in the hippocampus, cerebellum and thalamus; a visible band appearing from cDNAs from these regions after 24 cycles. After 26 cycles, PCR products could be detected from cDNAs of the cortex, olfactory bulb, nucleus accumbens, hypothalamus and CRI G1 cell line. Appearance of PCR product from the amygdaloid nucleus and striatum, and then eye cDNAs followed after 28 and 30 PCR cycles, respectively. No PCR

Table 1
PCR primers used for the amplification of specific cDNA products from reverse transcribed total RNA

Gene	Accession number	Nucleotides	Primer	Sequence
BIR1	X83583	5' untranslated 9-28	Forward	5'-GTT CCA AGA GAG GCG ATG AGG-3'
			Reverse	5'-GGC CAA GTT AAC TGA ATC CA-3'
			Reverse	5'-CAG GGT GGT GAA GAT GTC CG-3'
rcK _{ATP}	X83584	994-1014	Forward	5'-CGA TTC ACA CCA GTC CTC ACC-3'
			Reverse	5'-CAG CCC CCA AGC AAA GGA-3'
GIRK1	L25264	1174-1192	Forward	5'-GCA CCA GCC ATA ACC AAC-3'
			Reverse	5'-TTG CCA GGA ACC GAA CTT-3'
β -Actin	V01217	2508-2527	Forward	5'-GAG CTA TGA GCT GCC TGA CG-3'
			Reverse	5'-CAT TTG CGG TGC ACG ATG-3'
Sucrase-isomaltase	X15546	1167-1185	Forward	5'-GGG GAA GTG GAA CAC ACG G-3'
			Reverse	5'-AGC AGG AGT TGG CTG GAA TG-3'

All primers were synthesised on an Applied Biosystems 394 DNA/RNA synthesiser.

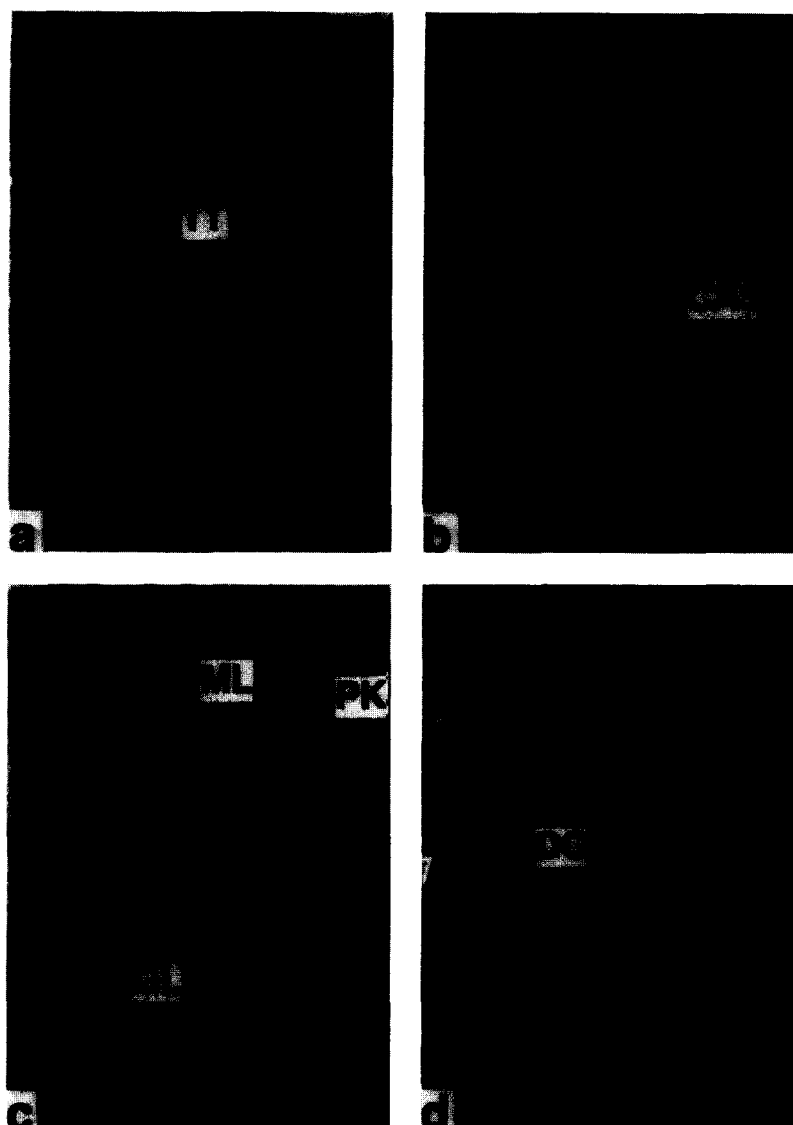


Fig. 4. Cellular expression of BIR1. Bright-field photomicrographs of sections of rat brain showing high densities of silver grains in the pyramidal cells of the taenia tecta (a), pyramidal cells of the CA3 region of the hippocampus (b), the granular layer of the cerebellum (c) and granular cell layer of the dentate gyrus (d): TT, taenia tecta; CA3, CA3 region of the hippocampus; ML, molecular cell layer; PK, Purkinje cell layer; GL, granular layer of the cerebellum; DG, dentate gyrus. Scale bar = 200 μ M.

product was visible from heart, skin and skeletal muscle following 34 cycles of amplification (data not shown).

Compared to the expression of BIR1, the tissue distribution of rcK_{ATP} mRNA was markedly different. Central expression of rcK_{ATP} was found to be prevalent in the hypothalamus and thalamus with only weak amplification detected from the cDNAs of other brain areas. As expected the highest level of amplification in peripheral tissues was detected from heart cDNA, and in addition, relatively strong expression was also detected in the eye and skeletal muscle. Low levels of amplification could be detected in the lung, uterus, bladder and CRI G1 cell cDNA whilst very little or no PCR product was obtained from spleen, stomach, jejunum, proximal colon, kidney, liver, testis and skin cDNA (Fig. 1). Furthermore, expression of GIRK1 was distinct from that of both BIR1 and rcK_{ATP} (Fig. 1). High levels of GIRK1 amplification were observed from cDNAs of all areas of the brain and in many of the peripheral

tissues: heart, lung, uterus, testis, eye, skeletal muscle and CRI G1 cells. Moderate levels of amplification were observed in all other peripheral tissues cDNAs, except for spleen cDNA from which no amplification was detected.

In situ hybridisation histochemistry was employed to elucidate the cellular localisation of BIR1 expression in the rat brain and confirm central distribution patterns observed from RT-PCR studies. Northern blot analysis of poly(A)⁺ RNA from whole rat brain was used to confirm the specificity of the antisense oligonucleotide probe, which hybridized to a single RNA band corresponding to the expected size of, 1.4 kb (Fig. 3.1). In situ hybridisation of the ³⁵S-labelled oligonucleotide probe BIR1 mRNA was then carried out on vertical, sagittal and horizontal sections of rat brain (Fig. 4). Hybridisation to BIR1 mRNA was greatest in the hippocampus, dentate gyrus, cerebellum and the taenia tecta (the anterior rudiment of the hippocampus). In addition, these studies revealed marked expres-

sion of BIR1 in the cortex, the habenular nucleus, the olfactory bulb, the primary olfactory cortex, the thalamus, the pontine nucleus and the amygdaloid nucleus (Fig. 3). Under identical conditions, no specific hybridisation of the sense oligonucleotide probe was observed (Fig. 3: 2d, 3d, 4f). To gain further insight into the cellular localisation of BIR1 expression, sections were dipped in photographic emulsion and subjected to microscopic examination. This demonstrated that all pyramidal cells of the taenia tecta (the anterior hippocampal rudiment) and of the CA1, CA2 and CA3 field of Ammon's horn in the hippocampus are strongly labelled (example of specific labelling, Fig. 4a and b). Within the cerebellum the medium-sized granule cells exhibit moderate levels of expression, specific hybridisation was not detected in Purkinje cells or the cells of the molecular layer (Fig. 4c). High expression was also observed in the granule cells of the dentate gyrus (Fig. 4d). Moderate levels of expression were also observed in the ventral tegmental area and the pars compacta region of the substantia nigra (the pars reticulata displayed little expression). This is in agreement with BIR1 being expressed in the dopaminergic cells of the pars compacta region of the substantia nigra (Henderson, G., personal communication). In regions where BIR1 is expressed at lower levels, e.g. cortex, the signal appeared to be generalised and not restricted to sub-populations of cells within these regions. Analysis of dipped brain sections revealed no detectable cellular expression of BIR1 mRNA in the striatum, possibly reflecting the limited sensitivity of this technique in comparison to RT-PCR.

4. Discussion

We describe herein the tissue and cellular localisation of mRNA expression of the inward rectifier K⁺ channel BIR1. The strong expression of BIR1 in many brain areas, the eye and the insulin secreting cell line CRI-G1, but not in any other peripheral tissue agrees with the original findings [1]. The distribution pattern of rcK_{ATP} from our RT-PCR tissue studies extends the observations of and is in general agreement with the published data [6] with the exception that we find a strong signal from skeletal muscle for rcK_{ATP}. The distribution pattern of GIRK1 however shows differences to that previously published. For example in situ hybridisation studies have demonstrated abundant expression of GIRK1 (KGA) in rat brain but no signal in lung, skeletal muscle or kidney [16]. Our RT-PCR data indicate that GIRK1 is widespread in rat brain in agreement with the former findings, but that the peripheral tissues examined including lung, skeletal muscle and kidney also display expression of this gene although in many cases at much lower levels. Analysis of BIR1 mRNA distribution in rat brain by in situ hybridisation revealed it to be present in many brain regions, and is particularly abundant in the granule cell layer of the dentate gyrus, all pyramidal cells in the CA1, CA2 and CA3 fields of Ammon's horn in the hippocampus, olfactory bulb, taenia tecta and the granule cell layer of the cerebellum. Thus there is a remarkable similarity in the distribution of BIR1 mRNA to that of GIRK1 mRNA in the central nervous system [16]. Clearly the widespread identity of distribution pattern for these inward rectifier sequences in rat brain suggests that they may also be linked functionally. Support for this comes from co-expression studies of cloned inward rectifier sequences. Recently BIR1 [11] and GIRK2 [10] have been shown to co-

assemble with GIRK1 and produce large G-protein-sensitive, inwardly rectifying potassium currents when expressed in *Xenopus* oocytes. In addition CIR, a protein identical to rcK_{ATP}, has also been shown to co-assemble with GIRK1 and enhance functional expression of G-protein gated currents [9]. Therefore heteropolymerization between GIRK1 and BIR1/GIRK2 or rcK_{ATP}/CIR may account for at least some of the diversity of inward rectifier K⁺ conductance's under transmitter/G-protein control described in central neurones. One surprising finding is the presence of GIRK1 mRNA in the CRI-G1 insulin-secreting cell line. Electrophysiological studies indicate no evidence for a muscarinic receptor-regulated G-protein sensitive inward rectifier in insulin secreting cells. However, GIRK1 may be a subunit of some other G-protein and transmitter regulated K⁺ channel in insulin secreting cells [17].

The relation is between BIR1 and K_{ATP} channels is unclear. rcK_{ATP} has been proposed as the cardiac K_{ATP} channel, or subunit thereof [6] and also as a component (CIR) of the endogenous muscarinic receptor linked G-protein-gated inward rectifier of cardiac atrial tissue [12]. The strong homology of BIR1 to rcK_{ATP} might indicate some similarity of function, however, expression studies of BIR1 mRNA in *Xenopus* oocytes and single channel analysis indicates that the currents induced do not behave as ATP-sensitive channels [1]. However, as outlined above evidence is rapidly accumulating that these proteins can heteropolymerize and form functional channels, and it is possible that BIR1 and rcK_{ATP} are components of the complex currently described as the K_{ATP} channel. Therefore, one explanation is that there are heterologous subunits present in various cell types which can polymerize or associate as subunits of these inward rectifiers and in so doing modify the physiological and pharmacological properties of the channel. Our demonstration that BIR1 is co-expressed in all areas of the CNS expressing GIRK1, suggests that such co-assembly is possible. One obvious candidate for a modifying subunit is the sulphonylurea receptor, recently cloned from rat pancreatic β -cells [18]. At present there are no reports of successful endowment of inward rectifier cloned sequences with glibenclamide sensitivity following co-expression of the sulphonylurea receptor in *Xenopus* oocytes. Consequently, either the inward rectifiers cloned so far bear no relation to the endogenous K_{ATP} channels or there are other heterologous subunits still to be identified. These data demonstrate a unique pattern of BIR1 expression in the rat, compared with related sequences, and shed light on possible interactions between the potassium channel subunits.

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